Diagnosis of Nasopharyngeal Carcinoma by DNA Amplification of EBV Genomes in Nasopharyngeal Biopsy and Fine-Needle Aspiration of Neck Nodes

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Abstract

Background
Nasopharyngeal carcinoma (NPC) is common in Malaysia but diagnosis is sometimes delayed for non-representative biopsy, submucosal disease and occult primaries - increasing morbidity and mortality Epstein-Barr virus (EBV) is associated with all types of NPC and DNA in tumor cells is detectable by polymerase chain reaction (PCR) EBV products EBNA1, EBNA2 and LMP1 are implicated in oncogenesis and is detectable in nodal tissue However no similar study has been done in Southeast-Asia with adequate sample

Objectives
This study evaluates the validity and reliability of detecting EBV genes in biopsy and FNAC tissue in NPC by PCR.

Methodology
Tissue from 72 nasopharyngeal biopsies were collected from consented patients 36 were positive and 36 negatives served as controls Tissue from 70 fine-needle aspirations were similarly obtained 35 belonged to NPC-positive patients, and 35 of other pathologies served as controls
DNA was extracted, amplified with forward and reverse primers for EBNA1, EBNA2, LMP1 genes and human β-actin gene as control, and detected by electrophoresis Cloned DNA from B95-8 cell line served as positive control
Histopathological-proven primary tumour and clinico-pathological criteria for neck nodes (clinically suspicious neck node with histopathologically-confirmed primary tumour) were used as gold standard

Results
35/36 positive nasopharyngeal biopsies and 35/36 negatives contained sufficient DNA EBNA1 gene was detected in 34/35 positive specimens but were undetected in the controls EBNA2 gene was detected in 31/35 positive specimens and in 2/35 controls LMP1 was detected in 32/35 positive specimens and in 4/35 controls ($P > 0.05$ by McNemar's test - i e no significant difference from histopathology) EBNA1 has the best sensitivity (97 1%) and specificity (100%) (Kappa = 0 97) One patient in the control group was positive for EBV DNA and developed NPC 1 year later Another patient with obvious nasopharyngeal tumour was negative on the 1st biopsy and confirmed on repeat biopsy 2 weeks later, but EBV DNA was detected in both specimens 35/36 metastatic NPC specimens contained sufficient DNA and one was excluded due to presence of second primary EBNA1 gene was detected in 30/34 nodes and 1/34 controls EBNA2 gene was detected in 29/34 nodes and none of the controls LMP1 gene was detected in 30/34 nodes and in 2/34 of controls ($P > 0.05$ by McNemar's test – i e no significant difference from clinico-pathological criteria for neck metastasis) A cut-off point of >0/3 genes offers the highest sensitivity (97 1%) and specificity (94 1%) (Kappa = 0 91)
All histological types of NPC contained EBV DNA

Conclusion
EBV DNA detection is reliable and accurate in diagnosing NPC On par with histopathology in detecting primary tumours, it also predicts the development of NPC On par with clinico-pathological criteria in detecting metastatic NPC, it is superior to fine-needle cytology and can suggest NPC in occult primaries

1 Introduction

Nasopharyngeal carcinoma (NPC) is a potentially curable disease if detected early However, many pitfalls in the detection of NPC may delay diagnosis, increase morbidity and mortality, and worsen the prognosis of the disease These include late presentation, late referral to the ENT surgeon, difficulties in interpreting biopsy material and the entity of the 'occult primary' This study evaluates DNA amplification by polymerase chain reaction (PCR) of Epstein-Barr virus (EBV) genes and its role in areas of dilemma in the diagnosis of NPC

1.1 Background

1 1 1 Carcinoma of the nasopharynx

Nasopharyngeal carcinoma (NPC) is an epithelial tumour that is rare in most parts of the world - the incidence rate being less than 1 per 100,000 persons per year It has a distinct geographical and population pattern, gravitating towards China, Southeast Asia, Africa, Canada, Alaska and Greenland Eskimos The highest incidence is observed in the Cantonese-speaking Chinese of Guangdong province in South China, with an incidence of 30-50 per 100,000 persons per year, and high frequencies are observed similarly in emigrant southern Chinese populations in South-east Asia.

NPC is the commonest head and neck cancer in Malaysia. In Peninsular Malaysia, the incidence of NPC is 365 cases per year or 5 4 per 100,000 population (Prasad and Rampal, 1992) This disease affects mostly the Chinese (14 6 per 100,000), followed by the Malays (1 3 per 100,000) and least of all, the Indians (0 5 per 100,000) The highest incidences fall in the states of Selangor (26 9 per 100,000), Pahang (12 3 per 100,000) and the Federal Territory (10 7 per 100,000) The incidence rate rises after the age of 20 and plateaus at 40 to 59 years without any further rise with increasing age The age-adjusted male female ratio is 2 8 to 1

NPC constitutes 85% of malignant tumours of the nasopharynx, followed by lymphomas and other rare tumours such as the adenocarcinoma, adenoid cystic carcinoma and malignant melanoma (Watkinson *et al*, 2000) NPCs are classified by the World Health Organization (WHO) into three types

1 **Type I - Keratinising squamous cell carcinoma (SCC)**, seen on light microscopy and subdivided into well-, moderately-, and poorly-differentiated,
2 **Type II - Nonkeratinizing carcinoma (NK)** which is undifferentiated and shows squamous origins only on electron microscopy - and immunohistochemistry, and
3. **Type III - Undifferentiated carcinoma (UC).**

All types of NPC are now regarded as variants of the squamous cell carcinoma. However, keratinising SCC (WHO Type I) is uncommon and the well-differentiated subtype is extremely rare In a study of 1800 cases of NPC in Malaysia, (Pathmanathan *et al*, 1995) found 17% to be WHO type I and only 0 2% to be well-differentiated

1.1 2 Epstein-Barr virus in NPC

The Epstein-Barr virus (EBV) is a double-stranded DNA virus of the *Herpesviridae* family, first described by Epstein *et al* in 1964 Apart from their infective illnesses, EBV is strongly associated with Burkitt's lymphoma, B-cell lymphoma, Hodgkin's disease, T cell lymphomas and NPC It may sometimes be found in lymphoepithelial carcinomas of the stomach, salivary gland, lung and thymus (Allen, 1999, Kanegane *et al*, 2002), and also with oral cancers in Japan (Shimakage *et al*, 2002)

Old *et al* first described the serological relationship between EBV and NPC in 1966 and it has been subsequently established that patients with NPC have elevated antibody levels against various EBV-associated antigens, correlating with stage and tumour burden. NPC cells were shown by Klein, *et al* (1974) to contain multiple copies of the EBV genome EBV genomes and associated antigens have consistently been found in undifferentiated and well-differentiated NPC (Raab-Traub *et al*, 1987) The strong and consistent association of EBV with NPC suggests that it may have an oncogenic role

Pathmanathan, *et al* (1995) using polymerase chain reaction amplification of cDNA to detect EBV transcription for EBNA1, LMP1 and LMP2 genes showed that all types of NPC, regardless of histology or differentiation, contain episomal EBV genomes and express specific EBV genes. Moreover EBV DNA was found in the preinvasive state of NPC Similarly Huang and Lo (1999) described the presence of EBV DNA in all forms of NPC as well as the precursor state of Nasopharyngeal Intra-Epithelial Neoplasia Type III

Figure 1 - Map of the complete Epstein-Barr virus genome showing the PCR target zones for EBNA1, EBNA2 and LMP1 genes

1 1.3 Polymerase Chain Reaction (PCR)

PCR can be used to detect and amplify specific nucleic acid sequences of a virus Targeted amplification of nucleic acid sequences provides dramatic increases in the number of copies as well as equivalent reduction in the complexity of the nucleic acid to be probed Also, the exponential amplification of PCR catalyzed by this biochemically simple cyclical process requires less than minutes per cycle obviating the need for *in vitro* cell culture of a virus which may take weeks to months These aspects of PCR allow ready detection of a single virus particle especially when a specific host cell cannot be cultured and in latent infections where active viral replication is substantially attenuated thereby obviating procedures requiring the detection of proteins (Kwok and Sninsky, 1999) Such is the case of the Epstein-Barr virus in NPC which establishes a latent infection in the epithelial cells of NPC Needle punch biopsies and aspirates of various types have been shown to provide sufficient material for PCR analysis (Kwok and Sninsky, 1989)

1.2 Problems in the diagnosis of NPC

1 2 1 The 'normal nasopharynx'

A nasopharyngeal mass seen on examination can be biopsied under direct vision, utilizing a flexible or rigid endoscope, and the diagnosis is made quite easily. However, patients suspected clinically as having NPC but whose nasopharynx appears normal, will need deep biopsies taken from multiple sites and both Fossae of Rossenmüller These include patients with persistent unilateral serous otitis media, metastatic nodal disease, raised EBV titres, or radiological evidence consistent with NPC Sometimes, serial and multiple biopsies need to be undertaken before a diagnosis is reached (Woo, 1999) A significant proportion (13 3%) have occult primaries at presentation and the primary tumour may remain undetected even after repeated biopsies of the nasopharynx (Prasad *et al*, 1983) Causes of false negatives are non-representative biopsies, submucosal disease, and failure to recognize individual malignant cells or small clumps of tumour (Allen, 1999)

1 2 2 Cytological evaluation

Fine-needle aspiration (FNA) of nodal disease in NPC is a useful tool and can spare the patient from open biopsy which worsens the long-term prognosis considerably (Cai *et al*, 1983) It can make the diagnosis of metastatic undifferentiated or differentiated squamous carcinoma, and other carcinomas (e g adenocarcinoma, small cell carcinoma, sinonasal carcinoma) It may even suggest nasopharyngeal carcinoma as the origin when the presence of clusters of cohesive tumour cells, medium-sized oval vesicular nuclei, prominent nucleoli, pale cytoplasm, mitotic figures and intermingling mature lymphocytes are seen (Chan *et al*, 1988) With the help of immunohistochemical staining, carcinomas can be differentiated from lymphomas and granulomatous diseases However, FNA has limitations, which include inadequate or non-representative specimen, poor cellular preservation and an inexperienced cytopathologist (Chang and Chan, 1999) A study in Singapore on 123 fine-needle aspirates of neck masses (Kaur *et al*, 1993) had a diagnostic accuracy of 82 6% and inadequacy rate of 12 2% In a retrospective study of 57 cases of metastatic carcinoma of the neck with unknown primary found that only in 19 cases (33 3%) could the primary site be found with a rigorous diagnostic algorithm of lymph node biopsy, rigid panendoscopy with systematic biopsies of suspect regions as well as blind biopsies of endoscopically inconspicuous regions, including the tongue base and nasopharynx and bilateral tonsillectomy (Haas *et al*, 2002) Chang & Chan (1999) proposes the detection of EBNA (Epstein-Barr virus nuclear antigen) or in-situ hybridisation of EBER (Epstein-Barr virus-encoded RNA) on histological and cytological material as 'a very useful adjunct in diagnostic pathology', especially when conventional immunohistochemical techniques are unhelpful

1.3 Review of the literature

Various studies have been done to ascertain the feasibility of detecting EBV DNA and RNA in the fine-needle aspirate of metastatic nodal disease of NPC A comparison of their results is shown in the Table 1 below

Table 1 - Comparison of various studies done to detect EBV genes in the fine-needle aspirate of metastatic nodal disease in NPC

Author	Year	Country	NPC patients	Controls	Sensitivity %	Specificity %
Ohshima *et al*	1991	Japan	4/4	5/32	100	78 1
Walter *et al*	1992	USA	15/18	0/17	88.9	100
Feinmesser *et al*	1992	Canada	9/9	2/137	100	98 5
Pacchioni *et al*	1994	Italy	7/7	0/18	100	100
MacDonald *et al*	1995	Canada	5/5	2/41	100	95 1
Smith *et al*	1995	USA	1/1	-	-	-
Akao *et al*	1996	Japan	3/3	-	-	-
Lee *et al*	2000	Taiwan	10/10	0/20	100	100

These studies have all been conducted outside of Southeast Asia. Their sample sizes have been small, ranging from 1 to 18 patients with confirmed NPC Although they demonstrate a consistently high sensitivity (88 9 – 100%) and specificity (78-100%), further study is needed to validate these findings with a larger sample size before PCR can be used as a diagnostic tool

In the eight studies reviewed above, two studies were done using Southern blot as the detection method (Ohshima *et al*, 1991, Smith *et al*, 1995) and Ohshima *et al* (1991) found that in cancers other than the lymphoepithelioma, only PCR could reliably detect EBV DNA Three other studies utilized PCR as the detection method (Feinmesser *et al*, 1992b, Macdonald *et al*, 1995, Walter *et al*, 1992) In-situ hybridization on tissue obtained from lymph nodes were used in another three studies (Akao *et al*, 1996, Lee *et al*, 2000, Pacchioni *et al*, 1994)

While Lee *et al* (2000) detected EBERs to indicate the presence of EBV, the other seven studies used EBV DNA. Feinmesser *et al* (1992b) used probes for EBNA1 and EBNA2 with much success

In three of these studies there were patients whose primary tumour was unknown at the time of sampling by FNAC but neck node tissue tested positive for EBV DNA. 2 out of 2 patients in the study by Feinmesser *et al* (1992b), 1 out of 2 in the study by MacDonald *et al* (1995) and 1 out of 1 in the study by Smith *et al* (1995) subsequently developed overt NPC which were confirmed histologically However, in none of these studies has the accuracy of detecting different genes been compared nor has the value of detecting multiple genes in combination been considered

Walter *et al* (1992) concludes that their study 'demonstrates the utility of EBV detection by the polymerase chain reaction in the evaluation of patients with metastases to neck nodes from occult primary carcinomas' Pacchioni *et al* (1994) in their study on undifferentiated NPC (UNPC) makes a similar conclusion that is 'detection of EBV in cervical metastatic adenopathy may be successfully used to identify the presence of occult UNPC' MacDonald *et al* (1995) says their results 'demonstrate the utility of NPC-diagnostic EBV gene amplification in FNA samples of neck metastases and suggest that the presence of the EBV genome in FNA samples of neck nodes is predictive of the presence of NPC.' Moreover, Feinmesser *et al* (1992) adds that 'the presence of EBV in metastases from an occult primary tumor is predictive of the development of overt nasopharyngeal carcinoma'

It is the objective of this study therefore to confirm the value of detecting EBV DNA as a diagnostic tool in NPC in this region. Using a larger-sized sample, this study will also attempt to compare the accuracy of EBV DNA detection of three different genes, and its value when taken in combination. Chapter 2 details the general and specific objectives of this study In Chapter 3, the exact methodology of patient selection, specimen collection, stratification, laboratory analysis and statistical method is outlined In Chapter 4, the results of this study are presented together with its validity data and statistical

significance Finally, in chapter 5, the importance and significance of the results are discussed and recommendations are presented in Chapter 6

2 Objectives

2.1 General objectives

2 1 1 To evaluate the amplification of EBV DNA in tissue as a diagnostic tool for nasopharyngeal carcinoma

2 1 2 To establish Malaysian data regarding the amplification of EBV DNA in tissue of nasopharyngeal carcinoma

2 1 3 To compare the detection rate of various EBV genes individually and in combinations, in the tissue of nasopharyngeal carcinoma

2.2 Specific objectives

2 2 1 To establish the validity of EBV DNA detection of three EBV genes (EBNA1, EBNA2 and LMP) in postnasal space biopsy and fine-needle aspirate of neck masses in NPC

2 2 2 To determine the diagnostic accuracy of EBV DNA detection of three paired-gene combinations in postnasal space biopsy and fine-needle aspirate of neck masses in NPC

2 2 3 To determine the optimal diagnostic cut-off value for the total number of genes detected in postnasal space biopsy and fine-needle aspirate of neck masses in NPC

2 2 4 To compare the accuracy of FNAC and PCR in the detection of metastatic nodal disease

2 2 5 To ascertain the association between EBV genes with histological type

3 Methodology

This is a study to evaluate the validity of DNA amplification of various Epstein-Barr viral genes compared to histopathological examination (HPE) as the gold standard

3.1 Population, period and place of study

3 1 1 Population Patients from the Otorhinolaryngology clinic of Hospital USM, Kelantan and Ear, Nose and Throat Clinic of Hospital Pulau Pinang

3 1 2 Period of study July 2002 to September 2003

3 1 3 Place of study Otorhinolaryngology Clinic of Department of Otorhinolaryngology and Head & Neck Surgery, USM, Ear, Nose, and Throat Clinic of Hospital Pulau Pinang, Microbiology Laboratory of Department of Microbiology, Histopathology Laboratory of Department of Pathology, School of Medical Sciences, Universiti Sains Malaysia (USM)

3.2 Sample size

3.2 1 Active

71 tissue specimens positive for nasopharyngeal carcinoma were selected for PCR analysis (36 primary tumour biopsy and 35 nodal metastasis fine-needle aspirates) from patients who fulfilled the inclusion criteria These specimens were grouped as

1 Group N (36) – proven NPC, postnasal biopsy of primary tumour in the nasopharynx
2 Group F (35) – proven NPC, fine-needle aspirate of metastatic neck node

3 2 2 Control

71 tissue specimens negative for nasopharyngeal carcinoma were selected (36 postnasal space biopsy and 35 neck mass fine-needle aspirates) from patients who fulfil the inclusion and exclusion criteria.

These specimens were grouped as

1 Group A (36) – proven non-NPC, postnasal biopsy
2. Group B (35) – proven non-NPC, neck mass fine-needle aspirate

3.2 3 Sample size calculation

Sample size was derived with a Power and Sample Size Program®†, based on the confidence limit of 0 95, type I error [α] of 0 05, power of study [1-β] of 0 9, type II error [β] of 0 1, odds ratio [ψ] of 171, and correlation coefficient [ϕ] (arbitrary) of 0 5

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The required sample size of 34 pairs for each group was reached (34 for postnasal space biopsy and 34 for neck mass FNAC)

3.3 Patient recruitment

The number of patients recruited into this study was less than the number of specimens as more than one specimen may have been procured from a single subject Postnasal biopsy and fine-needle aspiration were performed in patients with a primary tumour and neck metastasis Fine-needle aspirates were taken from each neck node if multiple clinically suspicious neck nodes existed

3 3 1 Inclusion criteria

- Patient with suspected nasopharyngeal carcinoma, with or without neck metastasis, undergoing postnasal space biopsy
- Patient with neck mass undergoing fine-needle aspiration – confirmed cytologically or histopathologically to be a head and neck cancer other than the lymphomas, a benign disease or normal nodal tissue

3 3 2 Exclusion criteria

- Patients with more than one head and neck malignancy,
- Patients who have been subjected to radiotherapy or chemotherapy, or
- Debilitated patients who are unable to tolerate biopsy, fine-needle aspiration or surgery

3 3 3 Consent

Informed consent was obtained before enrolling a patient into the study He or she would be made to understand the nature of this study as explained in the Information Sheet (Appendices B and C) and must voluntarily agree to be part of this research. A translator was provided and consent was taken in the presence of an impartial witness when the patient could not understand English or Malay

3 3 4 Proforma

The patients' data were recorded in a Proforma (Appendix D) designed for this study The data includes -

- Patient biodata
- Inclusion and exclusion criteria
- Tissue collection – type and date
- Tumour TNM staging
- Histopathological type and grade
- Laboratory analysis and results

3.4 Specimen collection

Tissue was taken pre-emptively during routine diagnostic biopsy of the nasopharynx or fine-needle aspiration of neck masses As the diagnosis was not known at the time of biopsy, tissue was stored and subsequently classified into the various groups (N, F, A, B) based on the histopathology report Patients in the active groups (N and F) may have had one or more specimens taken as part of routine diagnostic investigations - a biopsy of the primary tumour of the nasopharynx and/or a fine-needle aspiration of the metastatic neck node for each clinically suspicious neck node present Patients in the control groups (A, B) may have had one or more tissue specimens taken from them – either from the nasopharynx or from a palpable neck node of any pathology other than NPC or lymphoma.

3 4 1 Biopsy of the nasopharynx

Informed consent was obtained and a biopsy from an obvious growth or the Fossa of Rossenmuller under visualisation was performed with a rigid or flexible endoscope in the standard way (Fig 8) The main bulk of tissue was placed in formaldehyde to be sent for HPE and a small segment of tissue, 1-2mm in size was placed in a 1 5mL tube and stored for later analysis pending histopathological confirmation If the histopathological examination (HPE) proved to be positive for NPC, the patient was recruited into the study under group N and if negative the specimen was used as a control in group A

3 4 2 Fine-needle aspiration of neck masses

Informed consent was taken for each patient and fine-needle aspiration was performed for neck masses using a 23G needle, 20cc syringe and needle biopsy carrier in the standard way (Fig 9) The main bulk of aspirate was smeared onto slides for cytological examination and the remainder put into a 1 5mL tube and stored for later DNA analysis If the specimen belongs to a patient with confirmed nasopharyngeal carcinoma on biopsy of the nasopharynx, it is classified under group F Otherwise the specimen was classified under group B unless cytological examination shows lymphoma, in which case it is excluded (because certain lymphomas are known to contain EBV and can be diagnosed by FNAC alone)

3 4 3 Storage and transport

Tissue specimens in 1 5mL tubes were stored in a freezer at the clinic temporarily and then transported in an ice-box to the -20°C freezer at the soonest possible time

3.5 Laboratory analysis

3 5 1 Histopathological examination (HPE)

All specimens collected were sent for routine histopathological and cytological examination. The diagnosis obtained from the pathologist confirmed nasopharyngeal carcinoma or otherwise and determined how they were classified (N,F,A,B) PCR results were compared to the histopathological diagnosis as the gold standard

3 5 2 PCR Analysis

All 138 tissue specimens with sufficient DNA (human β-actin gene positive) from the active and control groups were subjected to PCR analysis This method involves three main steps

1 Extraction of total DNA from tissue specimens,
2 Amplification of total DNA by PCR, and
3 Detection of EBV DNA by agarose gel electrophoresis

PCR was performed at the Department of Microbiology, USM They were analysed for the presence of three EBV genes – EBNA1, EBNA2 and LMP1 – and the human β-actin gene to ensure sufficient amounts of DNA extraction

3 5 2 1 Primer design

Primers are short oligonucleotides made of T,A,G and C nucleotides used by DNA polymerase to initiate synthesis of new complementary strands of DNA Forward and reverse primers were designed for the Epstein-Barr virus genes EBNA1, EBNA2 and LMP and also for the human β-actin gene (Table 2)

Primers were custom-made according to specification by a commercial company[1] (Appendix G)

3 5 2 2 EBV DNA positive control

EBV DNA positive control was derived from a B95-8 cell line[2] EBV DNA from the B95-8 cell line was amplified with *Taq* polymerase PCR using primers designed specifically for EBNA1, EBNA2 and LMP1 The PCR products were then cloned into PCR cloning vector TOPO 2 1 (Invitrogen) (Fig 10) The positive clones were selected by PCR screening The presence of EBV gene was further characterized by restriction analysis. The positive EBV clones were then used as positive control in each PCR analysis

3 5 2 3 Extraction of tissue DNA

DNA from the tissue samples were extracted using the Nucleospin® tissue DNA extraction kit (Fig 11) (Appendix G) Approximately 30mg of tissue was digested in lysis buffer containing Proteinase K at 56°C overnight (Fig 12, 13) The digested tissue sample was passed through a silica column (Fig 14) Since DNA has a high affinity for silica, it binds to it, and pure DNA was eluted from the column using a low-salt buffer

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[1] Alpha DNA®

[2] Courtesy of Dr Peh, University Malaya

3 5 2 4 Amplification of EBV DNA by PCR

1) Forward and reverse primers for EBNA1, EBNA2, and LMP genes of the Epstein-Barr virus were used in the polymerase chain reaction. Probes for the human β-actin gene were also used for every set to confirm sufficient quantity of DNA available for PCR amplification.

3 5 2 5 Detection of PCR product by electrophoresis

The PCR product obtained was run on 1% agarose gel containing ethidium bromide at 10 volts/cm (Fig. 16, 17, 18) Electrophoresis separates DNA molecules based on size and charge in an electric field Agarose is a seaweed that, when boiled, forms a matrix of interlocking fibres and pores The PCR product is loaded into wells at one end and negatively charged DNA migrates towards the anode Smaller molecules will migrate at a higher velocity, resulting in a gradient based on molecular size Ethidium bromide binds to DNA and serves as a DNA staining agent Using an image analyser, DNA bands will fluoresce under ultraviolet light and an image is photographed using the image analyzer The expected product size is compared with a marker (Appendix G)

3.6 Data collection

All data were stored digitally using Microsoft Excel 2000 spreadsheet as a database

3.7 Statistical analysis

All statistical analyses were performed using the proprietary software MedCalc® ver 7 2 0 2†

3 7 1 Specific Objective 1 To establish the validity of EBV DNA detection of three EBV genes (EBNA1, EBNA2 and LMP) in postnasal space biopsy and fine-needle aspirate of neck masses, in NPC

3 7 2 Specific objective 2 To determine the diagnostic accuracy of EBV DNA detection of three paired-gene combinations in postnasal space biopsy and fine-needle aspirate of neck masses, in NPC

The detection rate for three EBV gene-pairs for active and control groups were tabulated, i e for

- EBNA1 & EBNA2
- EBNA1 & LMP1
- EBNA2 & LMP1

3 7 3 Specific objective 3 To determine the optimal diagnostic cut-off value for the total number of genes detected in postnasal space biopsy, in NPC

The detection rate for using different cut-off points total number of genes for active and control groups were tabulated, i e for

- Cut-off point – 1 or more genes (>0)
- Cut-off point – 2 or more genes (>1)
- Cut-off point – 3 genes (>2)

3 7 4 Specific objective 4 To compare the accuracy of FNAC and PCR in the detection of metastatic nodal disease

The results of FNAC in clinically positive neck nodes of patients with proven primary tumour of NPC were compared with the detection of EBV genes by percentages

3 7 5 Specific objective 5 To ascertain the association between EBV genes with histological type

Detection rates for each gene were calculated for each histological type – Type I, II and III – according to the WHO classification.

4 Result

4.1 Subjects

4 1 1 Postnasal space biopsy

In the period of study, tissue from a total of 86 postnasal space biopsies were collected and stored

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Upon histopathological confirmation of the 86 postnasal biopsies, 36 were positive for nasopharyngeal carcinoma and 50 were negative (of which the first 36 were selected for PCR analysis). During PCR analysis for human β-actin gene, one specimen from group N and one from group A was negative and duly excluded from the study The final number of specimens was for Group N - 35 specimens and Group A - 35 specimens
The breakdown of their histopathological/cytological diagnoses is as follows·

- Group N (35)
  - WHO type I – 4 (11 4%)
  - WHO type II – 3 (8 6%)
  - WHO type III – 28 (80%)
- Group A (35)
  - No malignancy – 32 (91 3%)
  - Chronic inflammation -3 (85 7%)

4 1 2 Fine-needle aspirate of neck masses

In the period of study, tissue from a total of 71 fine-needle aspirates were collected and stored Upon cytological and histopathological confirmation, 36 neck nodes belonged to patients with nasopharyngeal carcinoma (proven by postnasal biopsy) and 35 others had other diagnoses One specimen from group F was reported as a 'vascular tumor' and thus excluded on the grounds that it may have been a second primary (as per exclusion criteria) During PCR analysis for human β-actin gene, one specimen from group F and one from group B were negative and duly excluded from the study. The final number of specimens was Group F - 34 specimens and Group B - 34 specimens
The breakdown of their cytological diagnoses is as follows

- Group F (34)
  - Metastatic carcinoma – 23 (67 6%)
  - No malignancy – 4 (11 8%)
  - No diagnostic material or insufficient for evaluation – 4 (11 8%)
  - Atypical cells – 2 (5 9%)
  - Necrotic debris -1 (2 9%)
- Group B (34)
  - Metastatic CA (10) – from ethmoid CA (2), CA ovary (1), oral CA (3), Undifferentiated parotid CA (2) and unknown primary (2) – (29 4%)
  - No malignancy – 3 (8 8%)
  - Chronic inflammation – 2 (5.9%)
  - Cystic lesion – 1 (2.9%)
  - Lymphoid cells – 1 (2 9%)
  - No diagnostic material or insufficient for evaluation – 17 (50%)

4.2 Individual EBV gene detection for postnasal space biopsy

Table 2 – Validity data for individual EBV gene detection in postnasal space biopsy (N series)

	EBNA1	EBNA2	LMP1
Sensitivity	97 14%	88 57%	91 43%
Specificity	100 00%	94 29%	88 57%
Positive predictive value	100 00%	93 94%	88 89%
Negative predictive value	97 22%	89 19%	91 18%
Likelihood ratio	infinity	15 50	8 00
95% Confidence interval	-0 96% to 1 43%	-4 68% to 7 66%	-6.25% to 7 92%
X^2	0 0000	0 1667	0 0000
p value	1 000 (>0 05)	0 6831 (>0 05)	1.0000 (>0 05)
Kappa statistic	0 971	0 829	0 800

4.3 Individual EBV gene detection for neck mass fine-needle aspirate

Table 3 - Validity data for individual EBV gene detection in fine-needle aspirate of neck masses (F series)

	EBNA1	EBNA2	LMP1

Sensitivity	88 24%	85 29%	88 24%
Specificity	97.06%	100 00%	94 12%
Positive predictive value	96.77%	100.00%	93 75%
Negative predictive value	89 19%	87 18%	88.89%
Likelihood ratio	30 00	infinity	15.00
95% Confidence interval	-2 42% to 8 22%	0 70% to 8.70%	-4 21% to 9 23%
X^2	1 5000	4 1667	0 5714
p value	0 2207 (>0 05)	0 0412 (>0 05)	0 4497 (>0 05)
Kappa statistic	0 853	0 853	0 824

4.4 Paired gene detection in postnasal space biopsy

Table 4 - Validity data for three paired-gene combinations in postnasal space biopsy (N series)

	EBNA1 + EBNA2	EBNA1 + LMP1	EBNA2 + LMP1
Sensitivity	88.57%	91 43%	88 57%
Specificity	100 00%	100 00%	94 29%
Positive predictive value	100 00%	100 00%	93.94%
Negative predictive value	89.74%	92 11%	89 19%
Likelihood ratio	infinity	infinity	15 50
95% Confidence interval	-1 12% to 5 71%	-1 67% to 4.29%	-4 68% to 7 66%
X^2	2 2500	1 3333	0.1667
p value	0 1336 (>0 05)	0 2482 (>0 05)	0 6831 (>0 05)
Kappa statistic	0 886	0 914	0 829

4.5 Paired gene detection in neck mass fine-needle aspirate

Table 5 - Validity data for three paired-gene combinations in fine-needle aspirate of neck masses (F series)

	EBNA1 + EBNA2	EBNA1 + LMP1	EBNA2 + LMP1
Sensitivity	79 41%	82 35%	82 35%
Specificity	100 00%	97 06%	100 00%
Positive predictive value	100 00%	96 55%	100 00%
Negative predictive value	82 93%	84 62%	85 00%
Likelihood ratio	infinity	28 00	infinity
95% Confidence interval	2 99% to 11 59%	-0 61% to 11 11%	1 81% to 10.14%
X^2	6 1250	3 1250	5 1429
p value	0 0133 (<0 05)	0 0771 (>0 05)	0 0233 (<0 05)
Kappa statistic	0 794	0 794	0 824

4.6 Cut-off value analysis for total number of genes detected in postnasal space biopsy

Table 6 – Validity data for total number of genes detected in postnasal space biopsy (N series)

	>0/3	>1/3	>2/3
Sensitivity	97 14%	91 43%	88 57%

Specificity	88 57%	94 29%	100 00%
Positive predictive value	89 47%	94.12%	100.00%
Negative predictive value	96 88%	91.67%	89.74%
Likelihood ratio	8 50	16 00	infinity
95% Confidence interval	-3.03% to 6 67%	-4 94% to 6 22%	-1.12% to 5.71%
X2	0 8000	0 0000	2.2500
p value	0 3711 (>0 05)	1 0000 (>0 05)	0.1336 (>0 05)
Kappa statistic	0 771	0 857	0 886

4.7 Cut-off value analysis for total number of genes detected in fine-needle aspirate of neck masses

	>0/3	>1/3	>2/3
Sensitivity	97 06%	85 29%	79 41%
Specificity	94 12%	97 06%	100 00%
Positive predictive value	94.29%	96.67%	100 00%
Negative predictive value	96 97%	86 84%	82 93%
Likelihood ratio	16.50	29 00	infinity
95% Confidence interval	-4 83% to 4 83%	-1 58% to 9 66%	2 99% to 11 59%
X^2	0.2500	2.2857	6 1250
p value	0.6171 (>0 05)	0.1306 (>0.05)	0 0133 (<0.05)
Kappa statistic	0 912	0 824	0 794

Table 7 - Sensitivity, specificity and area-under-curve for ROC in cut-off points for FNAC

Criterion	Sensitivity(95%CI)	Specificity(95%CI)	Area under curve
> 0/3	97 1 (84 6- 99 5)	94.1 (80 3- 99.1)	0 956
> 1/3	85 3 (68 9- 95 0)	97 1 (84 6- 99 5)	0 912
> 2/3	79 4 (62 1- 91 3)	100.0 (89 6-100 0)	0.897

4.8 Comparison of FNAC and PCR

In 11 cases of NPC with neck metastasis, PCR was able to detect EBV DNA while FNAC failed to detect malignancy However in one case, FNAC showed metastatic carcinoma while PCR failed to detect any of the three genes

Table 8 – Comparison of EBV gene detection and various cytological findings in the fine-needle aspirates of neck nodes in patients with known nasopharyngeal carcinoma.

FNAC for neck nodes in NPC (n=34)	EBNA1	EBNA2	LMP1	Any 1 out of 3 genes
Metastatic CA 67 6% (23)	20/23 (87 0%)	20/23 (87 0%)	19/23 (82 6%)	22/23 (95 7%)
No malignancy (4)	3/4 (75%)	2/4 (50%)	4/4 (100%)	4/4 (100%)
No diagnostic material or insufficient for evaluation (4)	4/4 (100%)	4/4 (100%)	4/4 (100%)	4/4 (100%)
Atypical cells (2)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
Necrotic debris (1)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)
Total (34)	30/34 (88%)	29/34 (85 3%)	30/34 (88 2%)	33/34 (97 1%)

4.9 Comparison of histological type and EBV gene detection by PCR

Of the 35 specimens of NPC postnasal biopsy tissues, 4 were classified as WHO type I (keratinizing squamous cell carcinoma), 3 were WHO type II (non-keratinising squamous cell carcinoma) and the other 28 were WHO type III (undifferentiated carcinoma)
A comparison of the histological type and EBVgene detection by PCR is tabulated below

Table 9 - Comparison of histological type and EBV genes

	EBNA1	EBNA2	LMP1
WHO type I (4)	4/4	4/4	4/4
WHO type II (3)	3/3	3/3	3/3
WHO type III (28)	27/28	24/28	25/28

5 Discussion

5.1 Significance of results

35/36 positive nasopharyngeal biopsies and 35/36 negatives contained sufficient DNA. EBNA1 gene was detected in 34/35 positive specimens and in none of the controls EBNA2 gene was detected in 31/35 positive specimens and in 2/35 controls LMP1 was detected in 32/35 positive specimens and in 4/35 controls HPE failed to detect malignancy in one patient who had an obvious tumour of the nasopharynx but was only confirmed to be NPC on a second biopsy 2 weeks later EBV DNA was found in both specimens. Another patient in the control group was positive for EBV DNA and developed NPC within 1 year

35/36 metastatic NPC specimens contained sufficient DNA and one was excluded due to presence of second primary EBNA1 gene was detected in 30/34 nodes and 1/34 controls EBNA2 gene was detected in 29/34 nodes and none of the controls

5 1 1 Individual EBV gene detection for postnasal space biopsy

In the tissue obtained from postnasal space biopsy, detection of EBNA1 gene is considerably better than that of EBNA2 and LMP1, showing a high sensitivity (97 1%) compared to EBNA2 (88 6%) or LMP1 (91 4%), and an extremely high specificity (100%) compared to EBNA2 (94 3%) or LMP1 (88 6%) as shown in Table 4 All three detection rates have p values above 0 05 on McNemar's test (Table 4) - i e there is no significant difference between PCR and HPE This shows the EBV DNA detection to be on par with the gold standard of histopathological examination. This is supported by the Kappa statistic which shows an excellent inter-test agreement (0 97) for EBNA1 and good agreement (0 8-0 9) for the other two genes (Table 4) Comparing the ROC curves with one another, the results indicate that EBNA1 gene has the highest area-under-curve (0 99) and is significantly different from that of LMP1 ($p<0.05$), and no statistical difference is shown between EBNA2 and LMP1 ($p>0.05$) as shown in Table 5 and 6 EBNA1 gene, showing the highest sensitivity and specificity, Kappa value and area under the ROC curve, makes it the most suitable and specific gene for the diagnosis of NPC in the biopsy tissue of the postnasal space It is on par with the gold standard of HPE and can provide an adjunct or alternative to diagnosis when it is not forthcoming from histopathological examination

5 1 2 Individual gene detection for fine-needle aspirate of neck masses

In this study, a clinico-pathological criteria (i e clinically suspicious neck node with histopathologically-confirmed NPC of the primary site) is taken as evidence of neck metastasis In the fine-needle aspirate of neck masses, detection of EBNA1 and LMP1 genes are equally sensitive (88 3%) while EBNA2 is only slightly lower (85 3%) as shown in Table 7 However EBNA2 stands out in specificity (100%) while EBNA1 comes in second (97 1% - 1 false positive) followed by LMP1 (94.1% - 2 false positives) All three detection rates have p values above 0 05 on McNemar's test (Table 4) - i e there is no significant difference between PCR and clinico-pathological criteria The Kappa statistic in this set is consistently high (>0 8) for all three genes, with EBNA1 and EBNA2 showing equal and the highest level (0 853) of inter-test agreement with histopathology (Table 7) Comparing their ROC curves with one another, the results indicate that the detection of EBNA1 has the highest area-under-curve (0 97) and is significantly different from that of EBNA2 and LMP1 ($p<0.05$), but no statistical difference is shown between EBNA2 and LMP1 ($p>0.05$) as shown in Table 8 and 9 EBNA1 gene, showing the highest sensitivity, Kappa value and area under the ROC curve, makes it the most suitable single gene for the diagnosis of metastatic NPC in the fine-needle aspirate of neck masses. A slight compromise in specificity (97 1%) compared to EBNA2 (100%) should be acceptable However, EBNA1 gene detection (88 3% sensitivity) is superior to FNAC where the diagnostic accuracy is 67.6% in this study (Table 18) It becomes very valuable in suggesting NPC as the origin of occult primaries and can guide the clinician in the diagnostic workup

5 1 3 Paired gene detection in postnasal space biopsy

Taken in pairwise combinations (where two genes in a pair must both be detected to qualify as a 'positive'), the EBNA1-LMP1 combination seems to show the best sensitivity (91 4%) and specificity (100%) compared to the other two combinations which have lower sensitivities (88 6%) and equal or lower specificity (EBNA1-EBNA2 – 100%, EBNA2-LMP1 – 94 3%) – as shown in Table 10 . All three pairwise combinations have p values above 0 05 on McNemar's test (i e no significant difference from the gold standard of histopathological examination.) This indicates that pairwise-combinations are on par with the gold standard of HPE, supported by the Kappa statistic which shows that the EBNA1-LMP1 has an excellent inter-test agreement (0 91) followed by the EBNA1-EBNA2 combination (0 89) and EBNA2-LMP1 combination (0 83) – as shown in Table 10 The area-under-the-ROC-curve is best also for the EBNA1-LMP1 combination (0 96) but comparing the ROC curves, all three pairwise combinations show no significant difference from each other (p>0 05) – as shown in Table 11 and 12 Therefore, the EBNA1-LMP1 combination is the most suitable pairwise combination of genes for the diagnosis of NPC in postnasal space biopsy It is on par with HPE but not superior to the single gene detection of EBNA1 This can also be used as adjunct or alternative to diagnosis when it is not forthcoming from histopathological examination.

5 1 4 Paired gene detection in fine-needle aspirate of neck masses

The paired combination of EBNA2-LMP1 combination (where two genes in a pair must both be detected to qualify as a 'positive') shows the best sensitivity (82.4%) and specificity (100%) compared to the other two combinations which have equal or lower sensitivity and equal or lower specificity (Table 13) However, only the EBNA1-LMP1 combination shows a p value above 0 05 on McNemar's test (i e no significant difference from clinico-pathological criteria) The Kappa statistic is best for the EBNA2-LMP1 with the highest inter-test agreement with histopathology (0 82) followed by the other two combinations (0 79) The area-under-the-ROC-curve is best also for the EBNA2-LMP1 combination (0 91) but comparing their ROC curves, all three pairwise combinations show no significant difference with each other (p>0 05) as shown in Table 14 and 15 Therefore, while the EBNA2-LMP1 combination is the best of the three pairwise-combinations, it is not on par to the gold standard of histopathology and not superior to the single gene detection of EBNA1

5 1 5 Cut-off value analysis for total number of genes detected in postnasal space biopsy

Analysis of the cut-off point shows a reciprocal relationship where the sensitivity decreases and specificity increases with increasing cut-off point from >0/3 to >2/3 The >0/3 cut-off point gives the best sensitivity (97 1%) and lowest specificity (88 5%) while the >2/3 cut-off point has the highest specificity (100%) and lowest sensitivity (88 6%) – as shown in Table 16 All three cut-off points have p values above 0 05 on McNemar's test (i e no significant difference from HPE) The >2/3 cut-off point has the highest value for Kappa statistic (0 89) and area-under-ROC-curve (0 94) – as shown in Table 17 However, comparing there area-under-ROC-curves, there is no significant difference between the three cut-off points with one another (p>0 05)

For a disease such as NPC, it is desirable to use a cut-off point which offers the highest sensitivity so as not to miss detecting a potentially curable disease (while a high specificity is desirable in a disease with grave prognosis) Since there is no significant difference between the three cut-off points, it is arguably better to use the >0/3 or >1/3 cut-off points which offer better sensitivity (91 4-97 1%) It is on par with histopathological detection but not superior to single gene detection of EBNA1

5 1 6 Cut-off value analysis for total number of genes detected in fine-needle aspirate of neck masses

Analysis of the cut-off points in fine-needle aspirates show a similar reciprocal relationship between sensitivity and specificity The >0/3 cut-off point gives the best sensitivity (97.1%) and lowest specificity (94 1%) while the >2/3 cut-off point has the highest specificity (100%) and lowest sensitivity (79 4%) – as shown in Table 18 All three cut-off points have p values above 0 05 on McNemar's test (i e no significant difference from clinico-pathological criteria) The >0/3 cut-off point has the highest value for Kappa statistic (0 91) and also for the area-under-ROC-curve (0 93) – as shown in Table 18 and 19 However, comparing their area-under-the-ROC-curves, there seems to be no significant difference between the three cut-off points with one another (p>0 05)

Since >0/3 shows the best Kappa and area-under-ROC-curve values and offers the best sensitivity - which is ideal for a disease that is potentially curable - it is clearly the most suitable cut-off point in the diagnosis of metastatic NPC in fine-needle aspirate of neck masses In fact, this is on par with HPE and superior to EBNA1 single-gene detection and EBNA2-LMP1 paired gene detection. This makes it ideal for the detection of metastatic NPC, especially in occult primaries A summary table of the best detection methods for postnasal space biopsy and fine-needle aspirate of neck masses is presented in the table below

Table 10 – Comparison between single gene, paired gene and cut-off point for postnasal space biopsy and fine-needle aspirate of neck masses.

	Sensitivity	Specificity	Inter-test agreement (Kappa)	Area under ROC curve	p value (McNemar's)
N series - Postnasal space biopsy					
Single gene EBNA1	**97.1**	**100**	**0.97**	**0.99**	**>0.05**
Paired gene EBNA1-LMP1	91 4	100	0 91	0 96	>0 05
Cut-off point > 0/3	97.1	88 6	0 77	0.93	>0 05
F series – Fine-needle aspirate of neck mass					
Single gene EBNA1	88 3	97.1	0 85	0 93	>0.05
Paired gene EBNA2-LMP1	82 4	100	0 82	0 91	<0.05
Cut-off point >0/3	**97.1**	**94.1**	**0.91**	**0.96**	**>0.05**

In summary, for postnasal space biopsy tissue, single-gene detection using EBNA1 is superior to paired-gene or cut-off point analysis in diagnosing NPC. One patient in this study was not known to be a case of NPC at the time of biopsy but EBV DNA (EBNA2 and LMP1) was detected in the postnasal space biopsy tissue In follow up, this patient subsequently developed NPC within 1 year of the test This may be explained by the presence of EBV DNA in the preinvasive state as described by Pathmanathan *et al* (1995) or NPIN III (Nasopharyngeal intra-epithelial neoplasia stage III) as described by Huang and Lo (1999) In another case, biopsy failed to detect malignancy in an obvious tumour but subsequently confirmed it in a repeat biopsy 2 weeks later EBV DNA was found in both specimens

One must conclude that the detection of EBV genes is on par with HPE and that it can be an adjunct or alternative to diagnosis when the diagnosis is missed or when histopathological diagnosis is difficult for whatever reasons – e g non-representative biopsies, submucosal disease, technical difficulties in interpretation (Allen, 1999) It can also predict the development of NPC and therefore alert the clinician to perform serial biopsies and follow-up a patient very closely rather than dismissing him/her as normal with fatal results

As for fine-needle aspirate of neck masses, a cut-off point of >0/3 is superior to either single-gene or paired-gene detection at the expense of a slight reduction in specificity compared to single-gene detection using EBNA1 In this study, a clinico-pathological criteria (i e clinically suspicious neck node in the presence of histopathologically confirmed NPC at the primary site) is taken as confirmatory of NPC Barring open biopsy of a neck node, which worsens prognosis considerably (Cai *et al* , 1983), a clinico-pathological criteria is the best means of diagnosing metastatic NPC. One must conclude that the detection of EBV genes in fine-needle aspirate tissue is on par with existing clinico-pathological criteria and by far superior to FNAC in detecting metastatic NPC Therefore it is a valuable adjunct to guide the clinician in the diagnostic workup of occult primaries

6 Conclusion

The final result of this study is presented according to their specific objectives, together with their validity data and statistical significance These results have been obtained from an adequate sample size to achieve a power of study of 0 95

6.1 Individual EBV gene detection for postnasal space biopsy

The EBNA1 gene is the best gene for detection of NPC in postnasal space biopsy tissue and is statistically superior to EBNA2 and LMP1 genes It has a sensitivity of 97 1%, specificity of 100%, and inter-test agreement (Kappa) of 0 97 and is statistically on par with HPE as a diagnostic method

6.2 Individual gene detection for fine-needle aspirate of neck masses

The EBNA1 gene is the best gene for detection of NPC in fine-needle aspirate tissue of neck masses and is statistically superior to EBNA2 and LMP1 genes It has a sensitivity of 88 3%, specificity of 97 1%, an inter-test agreement (Kappa) of 0 85 and is statistically on par with present clinico-pathological criteria and superior to FNAC as a diagnostic method

6.3 Paired gene detection in postnasal space biopsy

The EBNA1-LMP1 pair is the best combination of 2 genes for the detection of NPC in postnasal space biopsy tissue but it is not statistically superior to the other 2 pair-wise combinations It has a sensitivity of 91 4%, specificity of 100%, an inter-test agreement (Kappa) of 0.91, and is statistically on par with HPE as a diagnostic method.

6.4 Paired gene detection in fine-needle aspirate of neck masses

The EBNA2-LMP1 combination shows the highest sensitivity (82 4%), specificity (100%), and inter-test agreement (Kappa) of 0 82 but it is not statistically equal to clinicopathological criteria. Therefore there is no valid pairwise combination for the detection of NPC in fine-needle aspirate of neck masses

6.5 Cut-off value for total number of genes detected in postnasal space biopsy

A value of >0/3 is the best cut-off point to take in the detection of NPC in postnasal space biopsy tissue as it offers the highest sensitivity (97 1%) for detecting a potentially curable disease At this cut-off point the specificity is 88 5% and inter-test agreement (Kappa) is 0 78, and it is statistically on par with HPE as a diagnostic method.

6.6 Cut-off value for total number of genes detected in fine-needle aspirate of neck masses

A value of >0/3 is the best cut-off point to take in the detection of NPC in fine-needle aspirate of neck masses as it offers the highest sensitivity (97 1%) for detecting a potentially curable disease At this cut-off point the specificity is 94 1%, inter-test agreement (Kappa) is 0 91, statistically on par with present clinico-pathological criteria and superior to FNAC as a diagnostic method.

6.7 Presence of EBV DNA in histopathologically 'normal nasopharynx'

The detection of EBV DNA is predictive of nasopharyngeal carcinoma even when histopathology is unable to detect malignancy in postnasal space biopsy tissue

6.8 EBV genes and histological type

There is no significant difference in the detection of EBV genes and histological type (according to WHO classification) in NPC

In summary, EBV DNA detection in tissue obtained from postnasal biopsy and fine-needle aspirate of neck masses is a relatively cheap, reliable and accurate method of diagnosing NPC, and

1) It requires minimal amount of tissue and is rapid
2) It is on par with histopathology in diagnosing NPC and on par with clinico-pathological criteria in detecting metastatic NPC
3) It is superior to fine-needle cytology
4) It can serve as an adjunct when histopathological diagnosis is missed and can predict the development of NPC

It can also suggest NPC and guide the clinician in the diagnostic workup of occult primaries

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